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# Polycyclic Aromatic Hydrocarbons Degradation by Alcanivorax Spp. Dominant Microbial Community from Beach Sediments: Implications for Bioremediation of Oil Polluted Marine Environment

Uzochukwu C. Ugochukwu [a,b].
[a] School of Civil Engineering and Geosciences, University of Newcastle Upon Tyne, NE1 7RU, United Kingdom
[b] SHELL/UNN Centre for Environmental Management & Control, University of Nigeria, Enugu Campus, Nigeria

**Abstract**
Biodegradation of crude oil polycyclic aromatic hydrocarbons by *Alcanivorax Spp*. dominant microbial community isolated from beach sediments in a site around St Mary's Lighthouse near Whitley Bay, Newcastle Upon Tyne, United Kingdom with geo-reference: N $55^0$ 04 ' 18", W $01^0$ 26 ' 59" was investigated. The polycyclic aromatic hydrocarbons (PAHs) studied were naphthalene, C1-C3 naphthalenes, fluorene, phenanthrene and dibenzothiophene. Gas chromatography-mass spectrometry was employed in the determination of the PAHs. Obtained results indicate that the microbial community degraded naphthalene, C1- naphthalene, C2-naphthalene and C3- naphthalene at 60, 72 , 90 and 42% biodegradation respectively whereas the three fused rings, fluorene, phenanthrene and dibenzothiophene were degraded at 23, 9 and 9% biodegradation respectively. However, it is not quite clear which bacterial genera was responsible for the biodegradation of the PAHs as we have not provided any evidence that *Alcanivorax Spp*. was solely responsible for the degradation of the PAHs.
**Keywords:** *Alcanivorax Spp*; Microbial community; Biodegradation; Polycyclic aromatic hydrocarbons; Crude oil

## 1. INTRODUCTION

Hydrocarbon pollution of land and especially marine environment due to oil spill is a major challenge facing the world today. The sustainability of the earth as a mega ecosystem would be under serious threat if the challenge posed by oil spillage is not controlled or overcome (Halpern et al., 2008). The occurrence of hydrocarbons in the environment as pollutants arises from anthropogenic and natural releases. It has been said that natural input from marine oil seeps alone is probably high enough to be able to cover the entire oceans in the world at a 20-molecular thick layer (Head et al., 2006). In 2010, the world recorded a huge anthropogenic release of hydrocarbons into the marine environment that remains unprecedented with about 600million Kg of crude oil released into the marine environment following the Deepwater Horizon explosion in the Gulf of Mexico (Crone and Tolstoy, 2010).

Crude oil is the most complex mixture of organic compounds and contains over 20,000 chemical components according to petroleomics studies (Marshal and Rogers, 2004). Despite this very extensive diversity in chemical composition, crude oil can be grouped into four operationally defined fractions namely, saturated hydrocarbons, aromatic hydrocarbons, resins and asphaltenes (Arske, 2002; Speight, 2007). Resins and asphaltenes are basically more polar and are therefore occasionally referred to as polar fractions. In terms of specific gravity (density), crude oil is classified into two major groups, light and heavy crude oil. Extensive biodegradation taking place in the petroleum reservoirs leading to the removal of mainly some of the saturated and aromatic hydrocarbons would ultimately cause an increase in the relative abundance of the polar fractions leading to the production of heavy crude oil. Light crude oil contains higher proportion of saturated hydrocarbons by mass and fortunately, saturated hydrocarbons are relatively easily biodegraded (Tissot and Welte, 1984). However, the less susceptible groups of compounds (aromatic hydrocarbons and the polar fractions) to biodegradation are more toxic and persistent in the environment and are therefore of more environmental significance (Head et al., 2006).

There are several natural physical, chemical and biological processes known as weathering that control the fate of crude oil in the marine environment. The most significant weathering processes associated with crude oil releases to the marine environment are spreading, evaporation, dissolution, dispersion, formation of water-in-oil emulsions, photochemical oxidation, adsorption onto suspended particulate matter, stranding on the shore line, sedimentation to bottom sediments and microbial degradation (Payne and McNabb, 1985; Neff, 1990; Wolfe et al., 1994).

Microbial degradation of crude oil hydrocarbon polycyclic aromatic hydrocarbons which is the focus of this study is a very important subject given its huge relevance to bioremediation of oil pollution in marine environment. Microbial degradation of crude oil hydrocarbons is only possible because under favourable

 

conditions, the microorganisms obtain their 'food' from the crude oil which acts as carbon and energy source to the microorganisms. Hydrocarbon degrading bacteria are prevalent in the environment including marine environment (Yakinov et al, 2007). There are several bacteria that can use crude oil hydrocarbons as carbon and energy source. In fact it is reported that there are over 175 genera of bacteria capable of using crude oil as sole carbon and energy source (Al-Malem et al., 2010).

It has been reported that the several genera of bacteria capable of degrading crude oil hydrocarbons actually have varied potentials towards the degradation of the hydrocarbon fractions hence, while some genera specialize in degrading alkanes, some others specialize in degrading aromatic hydrocarbons (McGenity, et al., 2012). Example, Several literature have reported that *Alcanivorax spp., Oleiphilus spp*., and *Thalassolituus spp.* degrade straight and branched alkanes whereas *Pseudomonas* s*pp.* and *Cycloclasticus spp*. degrade polycyclic aromatic hydrocarbons (PAHs) although there are reported cases of *Pseudomonas* s*pp.* degrading both alkanes and PAHs (Head et al., 2006; McKew et al., 2007; Teira et al., 2007; Niepceron et al., 2010).

*Alcanivorax spp* is well known for alkane degradation. Some studies have also reported the ability of these bacterial genera to degrade n-alkylbenzenes (Dutta and Harayama, 2001). However, its capability in degrading other groups of aromatic compounds found in crude oil such as PAHs is scarcely documented. This study reports the microbial degradation of crude oil PAHs by microorganism community that is predominantly *Alcanivorax spp*.

Major objectives are to determine the efficacy of Alcanivorax dominant microbial community found in beach sediments to degrade PAHs such as C1-C3 naphthalenes, fluorene, phenanthrene and dibenzothiophene.

## 2. MATERIALS AND METHODS

The source of the microorganisms employed in this study was beach sediments that were composed of sand particles collected in a sterilized glass bottle (Duran) from a site around St Mary's Lighthouse near Whitley Bay, Newcastle Upon Tyne, United Kingdom with geo-reference: N $55^0$ 04 ' 18", W $01^0$ 26 ' 59". The beach sediment samples were stored in cold room at 4 $^0$C prior to the experimental work. The nutrient source was Bushnel-Haas (BH) broth which was supplied by Sigma-Aldrich. The nutrient Agar and every other chemical used in this study were also supplied by Sigma-Aldrich. North Sea crude oil supplied by British Petroleum (BP) was the source of carbon and energy for the microorganisms employed in this study. In another study reported by Singh et al., (2009) with exactly same beach sediment as used in this study, the microorganisms were predominantly *Alcanivorax spp*.

### 2.1 Culture enrichment and isolation of active cells for biodegradation experiments

Whitley Bay sediments (20 g), 100 mL of BH medium (autoclaved) and 500 mg of crude oil were mixed in a 250 mL conical flask and incubated for 5 d while stirred continuously. Thereafter, the indigenous microbial cells which are predominantly *Alcanivorax spp*. were isolated by collecting 5 mL of the cell suspension for preparing a fresh subculture. Several subcultures were prepared until the ninth one which was finally harvested for the biodegradation experiments.

### 2.2 Biodegradation experiment

The test biodegradation experiment consisted of 5 mg/mL concentration of oil in BH medium (autoclaved) mixed with the cell suspension in a 100 mL serum bottle in which 10 mL of BH medium was used for providing the cells with nutrients. This experiment was carried out in triplicate and incubated for two months. The control experiment (without cells) was also carried out in triplicate and allowed to stand for same period of time.

### 2.3 Hydrocarbon extraction and analysis

Prior to extraction of the residual hydrocarbons after the biodegradation experiment, a surrogate standard (squalane) was spiked into the samples. Thereafter, three stages of extraction with 30 mL of dichloromethane (DCM) for each stage were employed to extract the residual hydrocarbons. The procedure of Bennett et al. (2002) was employed in separating the hydrocarbons from the polars. Before taking the samples for GC-MS analysis, 1,1-binaphthyl was spiked into the samples as internal standard. For the purpose of ensuring that there is no microbial degradation taking place in the control experiment, heptadecylcyclohexane was spiked into the control experiment as internal standard for quantitating the acyclic isoprenoids present in the oil such as phythane and pristane. The measured relative response factor (RRF) of the surrogate standard lied between an acceptable range of 0.7 and 0.8. The percentage recovery of the surrogate standard varied between an acceptable range of 70 and 120%. The selected polycyclic aromatic compounds studied were: naphthalene, isomers of methyl naphthalene, isomers of dimethyl naphthalene, isomers of trimethyl naphthalene, fluorene, phenanthrene and dibenzothiophene.

 

### 2.4 Gas Chromatography-Mass Spectrometry and Gas Chromatography-Flame Ionization Detector

Gas Chromatography-Mass Spectrometry was employed in the analysis of the PAHs in both the test and control experiments whereas Gas Chromatography-Flame Ionization Detector was mainly used in confirming that there was no biodegradation taking place in the control experiment by measuring the acyclic isoprenoids as described in section 2.3. The procedure for GC-MS and GC-FID analysis of the samples were as reported in Ugochukwu et al. (2014).

### 2.5 Determination of percentage biodegradation of the PAHs

The following equations were used to determine the percentage of the PAHs removed by biodegradation.

PAH as biodegraded (weight) = $B_c - B_t$.....1

PAH as biodegraded (%) = $[(B_c - B_t)/ B_c ] * 100$ .....2

Where:

$B_c$ = weight (mg) of the residual PAH for the control sample (without cells)

$B_t$ = weight (mg) of the residual PAH for the test sample (with cells).

### 2.6 Statistical analysis

Minitab version 17 was the statistical tool employed in this study for carrying out student t-test of the extent of microbial degradation of the analytes in which comparison is between any chosen two analytes with respect to their biodegradation. The statistical analysis is carried out at confidence interval of 95%.

## 3. RESULTS AND DISCUSSIONS

### 3.1 Removal of acyclic isoprenoids via microbial degradation

Biodegradation of crude oil hydrocarbons has been ranked by Peters & Modolwan (1993) from scale of 0-10 where the degree of biodegradation is described as undegraded, light, moderate, heavy, very heavy and severe. Crude oil biodegradation scales are based on the classes of hydrocarbons that are removed during biodegradation. There are several techniques that are employed in the assessment of the biodegradation of hydrocarbons (Wang *et al*., 2007). The most common ones involve the use of conserved internal standards. Conserved internal standards are mainly found amongst the biomarkers and are useful in assessing biodegradation because they are relatively resistant to microbial degradation (Prince *et al*, 1994; Wang *et al*., 2007). Examples of conserved internal standards useful in biodegradation of hydrocarbon studies are: pristane, phytane and hopanes (Wang *et a*l., 2007; Prince *et al*., 1994). The simplest approach in evaluating biodegradation at its earliest stage is to track the degradation of degradable hydrocarbons by reference to slowly degradable ones such as pristane and phytane where their ratios will give useful information about changes due to biodegradation (Prince *et al*, 1994). Hence, the ratios of nC17/pristane and nC18/phythane offer a quick assessment and indication of microbial degradation. For the control experiment, nC17/pristane and nC18/phythane ratios are 2.0 and 2.1 respectively indicating non-microbial degradation which is also evidenced by the preservation of the alkane peaks in the acquired chromatogram at the end of the incubation period (Figure 1a). These ratios are not measurable for the test experiment and the microbial degradation is evidenced by the disappearance of the alkane peaks (Figure 1b). The chromatogram is as shown in Figures 1a & b.

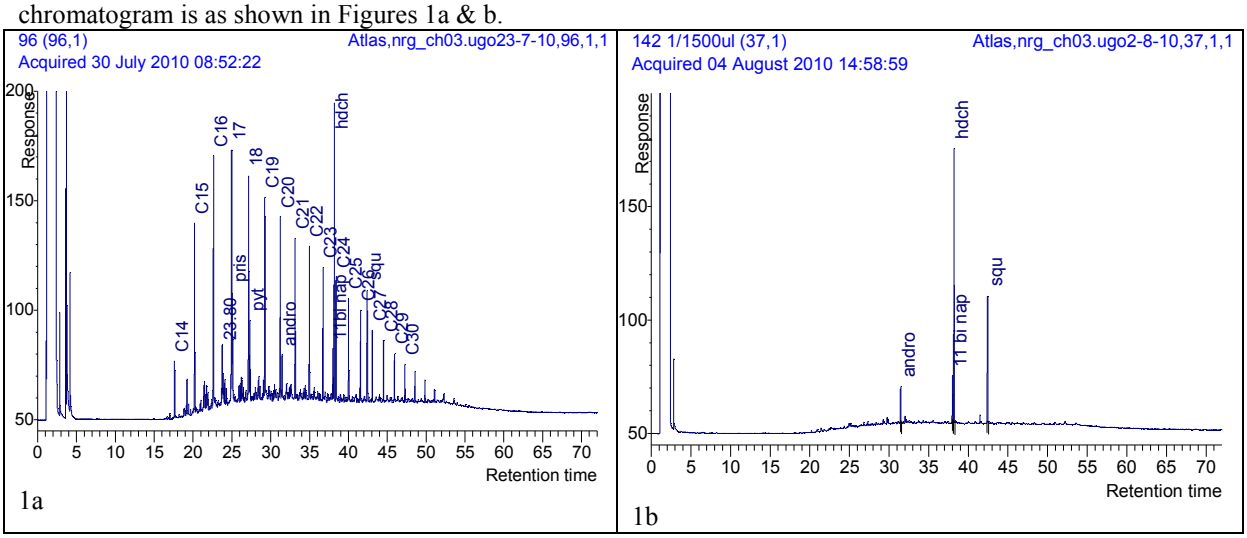


Figure 1 Chromatograms for test experiment (1a) and Control experiment (1b). Andro=androstane; hdch=heptadecylcyclohexane; squ=squalane; 1,1 Bi nap=1,1-binaphthyl.

 

### 3.2 Microbial Degradation of the PAHs

The percentage microbial degradation of the PAHs is as presented in Figure 2.

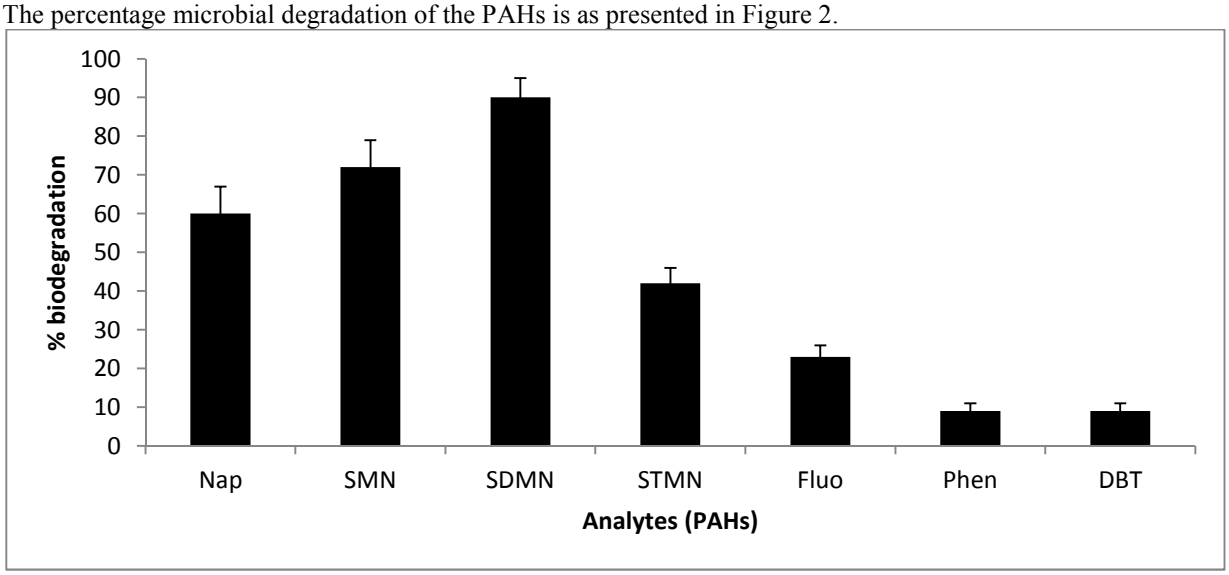


Figure 2: Percentage biodegradation of the PAHs. Nap = naphthalene; SMN = sum of the methylnaphthalene isomers; SDMN = sum of the dimethylnaphthalene isomers; STMN = sum of the trimethylnaphthalene isomers; Fluo = fluorene; Phen = phenathrene; DBT = dibenzothiophene. Values are reported as mean ± standard error of the mean.

The biodegradation of naphthalene, methyl- and dimethylnaphthalenes is significantly high at mean percentage biodegradation ranging from 60 to 91. Several studies have reported the biodegradation of naphthalenes, methylnaphthalenes and dimethylnaphthalenes with each indicating high microbial degradation ranging from 65 to 96% in most cases (Miyachi *et al*., 1993; Pawar *et al*., 2013; Karimi *et al*., 2015; Valentyne *et al*., 2015). In this study, there is no statistical significant difference between percentage biodegradation of naphthalene and methylnaphthalenes (P-value > 0.05 for each isomer). However, the difference in percentage biodegradation of naphthalene in comparison with the dimethylnaphthalenes is statistically significant (P-value < 0.05). The pattern of the biodegradation of these compounds appears very strange. It would have been expected that the lowest molecular weight compound among these three PAHs (naphthalene, methylnaphthalene and dimethylnaphthalene) and indeed all PAHs studied which is naphthalene would be the most biodegraded rather it is the highest molecular weight compound among the three, dimethylnaphthalene that is the most biodegraded. From previous studies, it has been reported that naphthalene is the most biodegraded among the three PAHs when considered as carbon source on individual basis and not in a mixture (McKenna and Heath, 1976). Recently however, it has been observed that there is preferential microbial degradation of methylated naphthalenes by some bacterial genera hence justifying the observed higher degradation of the methyl and dimethylnaphthalenes (Miyachi *et al*., 1993; Valentyne *et al.,* 2015). In this study, the percentage biodegradation of trimethylnaphthalenes at 42 is statistically significantly different from any of naphthalene, methyl- and dimethylnaphthalenes (P-value <0.05). McKenna and Heath (1976) observed that beyond two methyl groups on the naphthalene nucleus there would likely be reduced rate of oxidation of the PAH. It is therefore expected that trimethylnaphthalenes would undergo reduced microbial degradation in comparison with dimethylnaphthalenes or methylnaphthalenes. From previous studies, the main bacteria genera degrading the PAHs are *Cycloclasticus spp, mycobacteria spp., Pseudominas spp* etc. If we rule out the possibility of *Alcanivorax spp.* contributing to the degradation of the PAHs, then the only inference that could be drawn from this study is that the beach sediment employed in this study in addition to the *Alcanivorax spp.* contained other bacteria genera capable of degrading the PAHs with high preference to dimethylnaphthalenes.

### 3.3 Microbial Degradation of the isomers of the C1-C3 Naphthalenes

The percentage biodegradation of the isomers of methylnaphthalene, dimethylnaphthalene and trimethylnaphthalene is as presented in Figures 3-5.

 

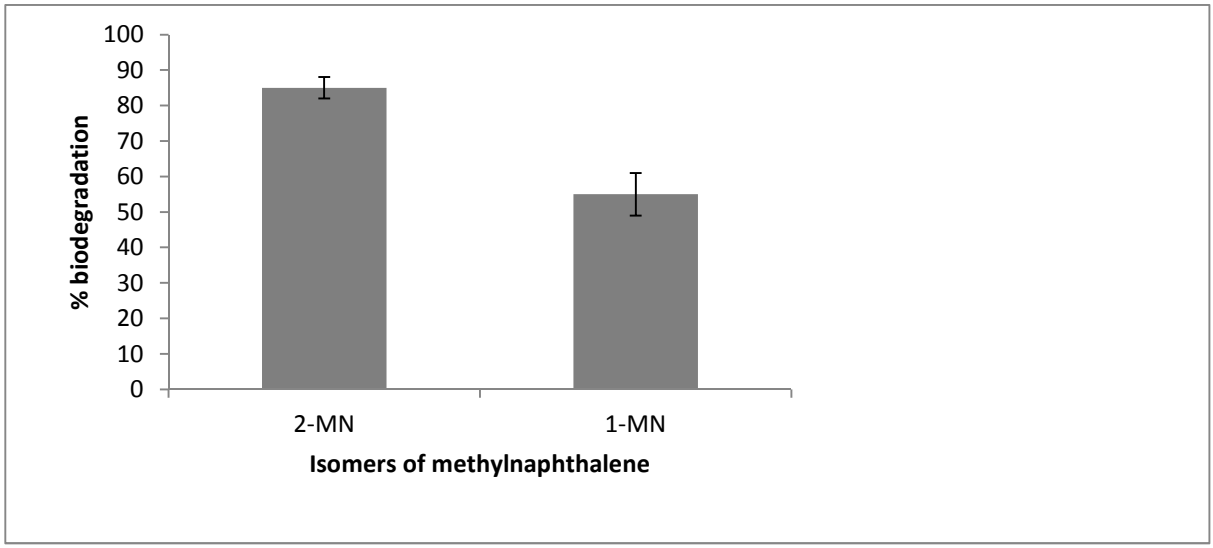


Figure 3: Percentage biodegradation of the isomers of methylnaphthalene. MN=methylnaphthalene. Values are reported as mean ± standard error of the mean

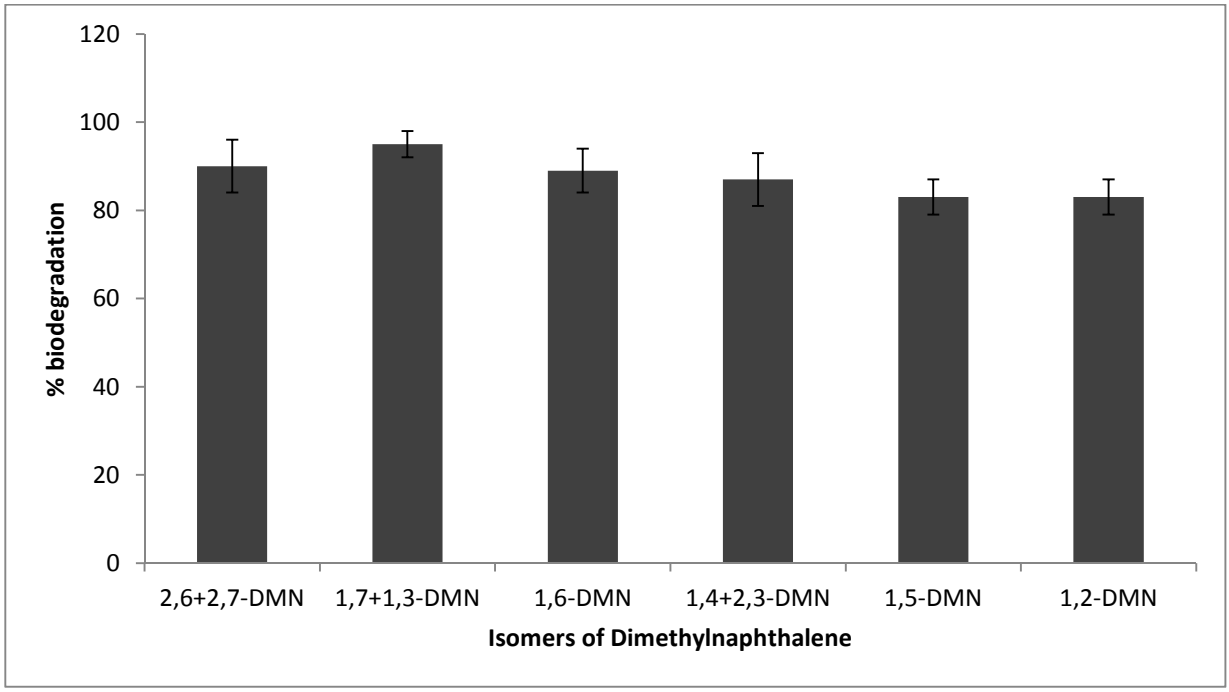


Figure 4: Percentage biodegradation of the isomers of dimethylnaphthalene. DMN=dimethylnaphthalene. Values are reported as mean ± standard error of the mean

 

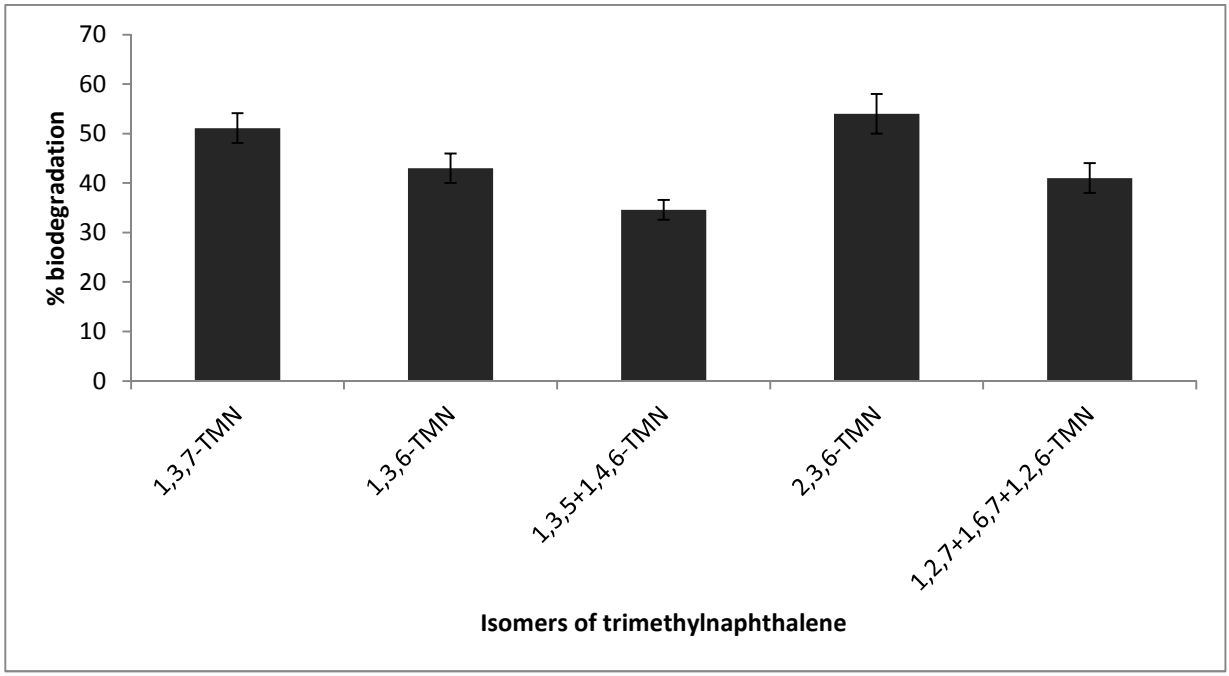


Figure 5: Percentage biodegradation of the isomers of trimethylnaphthalene. TMN=trimethylnaphthalene. Values are reported as mean ± standard error of the mean

There are two isomers of methylnaphthalene found in crude oil namely, 1- and 2-methylnaphthalene. In this study, the biodegradation of 2-methyl naphthalene is significantly different from that of 1-methylnaphthalene statistically (Figure 3; P-value <0.05). There are studies indicating that 2-methylnaphthalene is microbially degraded at a greater extent than 1-methylnaphthalene (McKenna and Heath, 1976; Valentyne *et al.,* 2015). Preference to 2-methylnaphthalene degradation has been attributed to steric hindrance which appears to affect this isomer to a less degree than 1-methylnaphthalene. It is not clear however, which microbial genera is responsible for this degradation.

The six isomers of dimethylnaphthalene studied in this work were degraded to a high degree and there is no significant difference statistically between any of the two isomers with respect to their microbial degradation (Figure 4). All the isomers lent themselves to substantial extent of biodegradation with percentage biodegradation of all the isomers ranging from 80 to 95% (Figure 4). It has been observed that methylating the naphthalene nucleus appears to aid the microbial degradation process most probably by promoting the interaction between the methyl group of the methylated naphthalene and the non-polar moiety of the cell membrane of the bacteria (Valentyne *et al.,* 2015). This being the case, this interaction will play out in the biodegradation of C1-C3-naphthalenes. If this interaction is the dominant factor determining the rate and extent of biodegradation, then C3-naphthalenes (trimethylnaphthalenes) would be the most biodegraded but this study demonstrated otherwise indicating that there could be other factors playing out to militate against the biodegradation of the mathylated naphthalenes at more than two methyl groups on naphthalene nucleus.

The extent of the biodegradation of either 2,3,6-trimethylnaphthalene or 1,3,7-trimethylnaphthalene is significantly higher than that of 1,3,5 + 1,4,6-trimethylnaphthalene statistically (Figure 5). It is not clear why the microbial organisms would accord higher preference to these two isomers. The biodegradation of the isomers of trimethylnaphtalene ranged from 35 to 54% which is quite lower than that of the biodegradation of the isomers of dimethylnaphthalene (Figures 4 and 5). Despite the increase in the number of methylene group attached to the naphthalene nucleus which is expected to bolster the biodegradation process of the trimethylnaphthalenes by promoting interaction with microbial cell membrane, the adverse effect of steric hindrance might be a predominant factor hence reducing the rate and extent of biodegradation of the trimethylnaphthalenes.

### 3.4 Microbial degradation of the three-fused-ring PAHs

The three-fused-ring PAHs studied in this work namely fluorene, phenanthrene and dibenzothiophene were not significantly biodegraded (Figure 2). This study revealed that the biodegradation of phenanthrene is not significantly different from that of dibenzothiophene statistically at average extent of biodegradation of 9% each case (Figure 2). Although the percentage biodegradation of fluorene at 23% is low, it is significantly higher than that of either phenanthrene or dibenzothiophene. It is suggested that the chemical structure of fluorene with a non-aromatic ring sandwitched in between two aromatic rings is likely to lend itself to a relatively higher rate of biodegradation than either phenanthrene or dibenzothiophene which have all three fused rings being aromatic.

 

Generally speaking, microbial organisms that degrade PAHs find it more difficult to degrade the PAHs as the fused rings increase hence it is not surprising that the biodegradation of the two fused ring PAHs in this study far outstrips that of the three fused ring PAHs. Under appropriate enabling environment, the microbial organisms can however be stimulated to enhance their ability to degrade even the three fused ring PAHs. Recently, several research studies have revealed that clay minerals have the ability to provide huge surface area in addition to sorbing toxic substances in the biodegradation system and as a result allow the proliferation of the microorganisms that in-turn feast on the PAHs in large numbers (Chaerun and Tazaki, 2005; Warr *et al*., 2009; Ugochukwu *et al.*, 2014; Biswas *et al*., 2015). The provision of enabling environment for the microbial community of the beach sediment would translate to an improvement on the degradability of the three fused ring PAHs present in the crude oil. This could be implemented during remedial operation of oil polluted marine environment.

## 4. CONCLUSION

This study having investigated the crude oil PAH biodegradation capability of the *Alcanivorax spp* dominant microbial community isolated from beach sediments demonstrated that even though the three-ring PAHs such as fluorene, phenanthrene and dibenzothiophene were not significantly degraded, the two-ring PAHs, naphthalene and C1-C3 naphthalenes were. However, the extent of biodegradation of trimethylnaphthalene (C3-naphthalene) was significantly lower than any of naphthalene, methylnaphthalene and dimethylnaphthalene. However, this study is not conclusive as further molecular biology is required to ascertain the bacterial genera that were most likely to be responsible for the degradation of the PAHs. Even though the beach sediment which was the source of the microbes was predominantly *Alcanivorax spp.* it is not clear whether the degradation of these PAHs was carried out solely by *Alcanivorax spp* or any other bacteria genera present in the oil. *Alcanivorax spp* is commonly known to specialize in the degradation of saturated hydrocarbons but in this sediment whereby it is the predominant genera, the degradation of low molecular weight PAHs has been observed. This study therefore has demonstrated that this microbial community isolated from beach sediments contains bacterial genera that are capable of degrading low molecular weight PAHs. This has a profound implication on the development of bioremediation strategies for the remediation of crude oil hydrocarbon contamination in marine environments.

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